Cell-Mediated Immunity after Intratracheal Exposure to 3-Methylcholanthrene, and its Relationship to Tumor Transplant Growth in C3H/f Mai Mice

Charles F. Demoise, Richard E. Kouri, and Carrie E. Whitmire

Department of Experimental Oncology, Viral-Chemical Carcinogenesis Section, Bethesda, MD 20014, USA

ABSTRACT

Immunological deficiencies have often been observed to occur in association with cancer although the exact nature of this relationship has not been fully characterized. The relative immunocompetence of an individual definitely plays a major role in the ultimate susceptibility or resistance to cancer. Numerous studies support the concept that cell-mediated immunity (CMI) is largely responsible for the body's defense against cancer. Our laboratory is currently interested in the levels of chemicals at which tumorigenesis occurs in various strains of mice and whether immunocompetence of the animals is affected.

In this investigation C3H/f Mai mice were intratracheally instilled 4 times at weekly intervals with 500 µg of 3-methylcholanthrene dissolved in a corn oil vehicle. These treatments caused 8% lethality in 30 days; whereas vehicle alone is nontoxic. Effects on CMI were determined 3 days after each treatment by measuring rates of DNA synthesis with ^{3}H-thymidine in allogeneic and spleen lymphocyte cultures. Spleen, thymus, and lung weight as well as blood leukocyte counts were measured. Syngeneic and allogeneic tumor transplants were performed on control and test mice to determine whether CMI data is biologically relevant to the process of tumor growth. The CMI and tissue responses were again evaluated 7, 14, and 28 days after tumor transplantation.

Preliminary data indicates that CMI, as reflected in spleen lymphocyte responses to phytohemagglutinin, pokeweed mitogen and allogeneic antigen, was suppressed during intratracheal instillations of 3-methylcholanthrene. This effect was most pronounced in response to pokeweed mitogen and persisted at least 2 weeks after exposures were discontinued. Lymphocyte cultures from mice that received tumor transplants indicate that the earlier CMI inhibition produced by carcinogenic exposure is not only cancelled but actually enhanced although only syngeneic transplants were successful. Again, it will be of interest to follow the kinetics of this effect in the host and compare it to the rate of tumor transplant growth.

A. Introduction

The intratracheal instillation of polycyclic hydrocarbons in hamsters, mice, and rats has served as a useful model for studies of respiratory carcinogenesis (SAFFIOTTI et al., 1968; NETTESHEIM and HAMMONS, 1971; SCHREIBER et al., 1972; SAFFIOTTI, 1969). Our laboratory is currently interested in the physiological effects of some of these chemical

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carcinogens, intratracheally instilled at concentrations known to induce respiratory malignancies. Evidence has accumulated during the past 15 years that clearly indicates the important role of host immunity in controlling the onset and progression of malignant diseases (MORTON, 1974). In this study as in others, we are characterizing some of the effects of chemical carcinogens on levels of host immunocompetence and their relationships to tumorigenesis.

B. Materials and Methods

I. Animals

Male C3H/f Mai mice 8 weeks old (Microbiological Associates, Walkersville, Maryland) and C57BL/6 Cum mice (Cumberland View Farms, Clinton, Tennessee) of similar age and sex were kept in disposable plastic cages containing corn cob bedding. They were given drinking water containing tetracycline (1 g/liter) and Purine Laboratory Chow ad libitum. A 12-hour lighting cycle was used.

II. Intratracheal Instillation of 3-Methylcholanthrene

C3H/f Mai mice were intratracheally instilled with 3-methylcholanthrene (MCA) according to the technique of HO and FURST (1973). Metofane (Pittman-Moore, New Jersey), an inhalation anesthetic, was used to anesthetize the mice. MCA (500 µg) was dissolved in 0.02 ml of corn oil (CO) and instilled in each of 100 test animals with a 19-gauge blunt needle attached to a Hamilton microliter syringe. A total of 4 such dosages were administered at 1 week intervals. Control mice (100) received only 0.02 ml CO.

III. Transplantation of Tumor Cells

Single-cell suspensions of syngeneic and allogeneic tumor cells from tissue cultures of a MCA induced C3H/f Mai tumor (passage 2) and a spontaneous BALB/c tumor (passage 2)(obtained from Dr. R. MADISON, Microbiological Associates) were diluted in Hanks' Balanced Salt Solution (1×10^7 cells/ml) for transplantation. Syngeneic and allogeneic tumor cells (1×10^6/0.1 ml) were inoculated into both MCA and CO exposed mice 2 days after the fourth, and final, intratracheal instillation. These were inoculated subcutaneously over the forehead for ease of palpation and measurement of growth.

IV. Culture Media, Mitogens, and Allogeneic Antigen

The culture media used was RPMI No. 1640 from Microbiological Associates supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin, 100 µg/ml of streptomycin and 200 mM of L-glutamine. Phytohemagglutinin-M (PHA) and pokeweed (PW) mitogens purchased from Difco (Detroit, Michigan), and Gibco (Grand Island, New York), respectively, were reconstituted in sterile-distilled water and used at a final concentration of 1% v/v in culture media. Freshly prepared single-cell suspensions of spleen cells from C57BL/6 Cum mice were exposed to 4,000 R of X-irradiation and served as a source of allogeneic antigen.

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V. Spleen Lymphocyte Culture

The relative reactivities of thymus-derived (T) and bone marrow-derived (B) spleen lymphocytes were distinguished *in vitro* by their responses to mitogenic challenge with PHA and PW respectively (ANDERSSON et al., 1972). T-cell activity was further assessed in the mixed lymphocyte culture (MLC) reaction in response to stimulation by allogeneic antigen (C57BL/6 Cum spleen cells)(PLATE and MCKENZIE, 1973).

Single-cell suspensions of spleen lymphocytes obtained from 5 individual mice sacrificed at regular intervals after each intratracheal instillation of MCA (3 days post-exposure) and at 7, 14, and 21 days after transplantation of tumor cells were prepared in the same manner for all *in vitro* assays of cell-mediated immune (CMI) activity. Spleens from MCA and CO mice were removed, their capsules opened with scissors, and the cells teased out into chilled media. The suspension was allowed to settle briefly to permit removal of connective tissue fragments and large cell clumps. After 3 centrifugations (1,000 rpm for 10 min) and rinses, the single-cell suspension of spleen lymphocytes was adjusted to a density of 6×10^6 cells per ml of media. For mitogen stimulated cultures, spleen lymphocytes (6×10^5/1.2 ml) were pipetted in quadruplicate aliquots into wells of Falcon No. 3040 plastic microtiter plates. PHA and PW were then added to give a final concentration of 1% v/v in each well. Media alone was used for unstimulated controls.

The assay for MLV activity was also set-up with 6×10^5 spleen lymphocytes per 0.2 ml of media per well from MCA or CO mice. Syngeneic (C3H/f Mai) and allogeneic (C57BL/6 Cum) X-irradiated spleen lymphocytes (6×10^5 cells in 0.2 ml media) were used for antigenic stimulation.

Both mitogenic stimulated and mixed lymphocyte cultures of spleen cells were incubated at 37°C for 48 hours in a 5% CO_2 atmosphere. One micro Curie of ^{3}H-thymidine was then added to each culture and incubated an additional 18 hours. After a total of 66 hours incubation, the cultures were harvested and the contents of each well were transferred to DEAE Whatman No. 81 filter pads and allowed to dry. These filters were washed 4 times with 5% dibasic sodium phosphate, followed by 5 washes with distilled water, and again dried. Filters were transferred to vials containing 10 ml of Liquifluor (Beckman Instruments, Fullerton, California) and counted in a Beckman model LS 250 scintillation counter. Responses of T and B populations of spleen lymphocytes were expressed as the difference in counts per minute (ΔCPM) between the unstimulated and mitogen stimulated cultures. Similarly, in the MLC reaction, the response of T cells was expressed as the difference in counts per minute (ΔCPM) between syngeneic and allogeneic antigen stimulated cultures.

VI. Host Tissue Measurements

Animal weights were obtained on all mice at weekly intervals and at times when mice were sacrificed for spleen lymphocyte cultures. Pooled thymuses and individual lung and spleens were weighed. The total number of peripheral blood leukocytes was obtained using a Fisher Autocytometer II (Fisher Instruments, Pittsburgh, Pennsylvania).

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C. Results

I. Effects of MCA Exposure and Tumor Transplantation on Animal Weight

Animal weights of CO control and MCA test mice recorded after each intratracheal instillation and after transplantation of syngeneic and allogeneic tumor cells are shown in Fig. 1. After 4 exposures and 24 days on test, MCA-mice showed a 10% loss of weight compared to the CO controls. This effect persisted even after intratracheal instillations were discontinued, although by day 40 of the mice previously exposed to MCA did gain weight. It is interesting to observe that mice receiving syngeneic transplants (5 days after the last MCA treatment) unterwent a pronounced loss of weight through day 46, 20 days after tumors were transplanted. As presented later, syngeneic cells grew but allogeneic tumor cells did not.

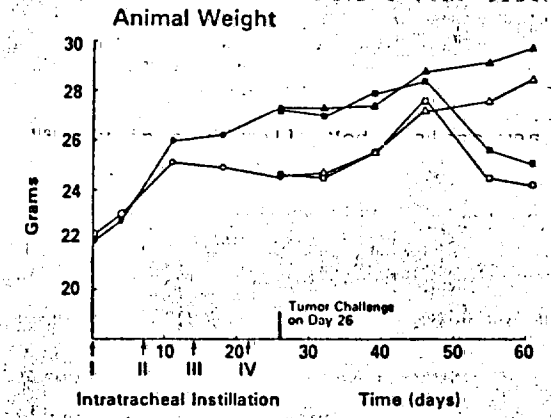

Fig. 1. Weight of mice in relation to intratracheal instillation of corn oil (solid circles) or 3-methylcholanthrene (open circles) and subsequent transplantation of syngeneic (squares) or allogeneic (triangles) tumor cells on day 26. Arrows indicate times of intratracheal instillation at 0, 7, 14, and 21 days.

II. Effects of MCA on Host Tissue Response

Table 1 shows the effects of MCA exposure on thymus, lung, and spleen weights and on peripheral blood leukocyte counts. All of these tissues were noticeably altered. Loss of thymus weight ranged from -38% after 1 exposure to -60% after 4, whereas lung weight increased as a function of MCA exposure. Spleen weights were depressed during MCA instillation and rebounded after they were discontinued. Leukocyte counts appeared to have increased as a function of MCA instillation.

III. Growth of Tumor Transplants

Syngeneic and allogeneic tumor cells were transplanted in CO control and MCA test mice, but only the syngeneic transplants grew into tumors, as indicated in Table 2. Tumors were first detected by palpation in both CO and MCA mice at 10 days after transplantation. It is interesting that tumors in MCA-exposed mice were significantly ($p < 0.01$) smaller than those in CO mice at 13 days and were also somewhat smaller at 28 days after transplantation.

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Table 1. Response of host tissues to 3-methylcholanthrene exposure[a]

Days on Test	No.[b] of Rx	ORGAN WEIGHT (grams) THYMUS C O	THYMUS MCA	THYMUS % Change	LUNG C O	LUNG MCA	LUNG % Change	SPLEEN C O	SPLEEN MCA	SPLEEN % Change	TOTAL CELLS (x 10^3) LEUKOCYTES C O	LEUKOCYTES MCA	LEUKOCYTES % Change
3	I	.047	.029	−38	.052	.069	+11	.065	.057	−12	3.2	2.9	− 9
10	II	.047	.028	−40	.228	.243	+ 7	.104	.088	−15	6.0	5.7	− 5
17	III	.055	.039	−29	.224	.250	+12	.068	.059	−13	5.0	6.0	+20
24	IV	.055	.022	−60	.214	.236	+10	.064	.061	− 5	4.0	7.0	+75
28	IV	.044	.027	−39	.186	.215	+16	.054	.070	+30	6.7	7.6	+13
35	IV	.033	.034	+ 3	.162	.191	+18	.064	.084	+31	6.6	8.0	+21

[a]Weights and cell totals represented as arithmetic mean of 10 mice

[b]Cumulative number of intratracheal instillations of 500 ug 3-Methylcholanthrene (MCA) in 0.02 ml corn oil (C O)

Table 2. Syngeneic and allogeneic tumor growth in 3-methylcholanthrene exposed mice

Tumor Type[a]	Treatment[b]	Tumor Size (cm) at Days After Transplantation 10	13	28	Tumored Mice No. Tested	(%)
Syngeneic	C O	palpable	0.94	1.91	26/26	(100)
	MCA	palpable	0.75	1.79	26/26	(100)
Allogeneic	C O	no tumor	0.00	0.00	0/26	(0)
	MCA	no tumor	0.00	0.00	0/26	(0)

[a]1×10^6 syngeneic (C3H/fMai) or allogeneic (BALB/c) tumor cells were injected subcutaneously in mice five days after the fourth and final intratracheal instillation

[b]Corn oil (C O) alone or 500 ug 3-Methylcholanthrene (MCA) dissolved in C O were administered intratracheally four times at weekly intervals before tumor transplantation

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IV. Cell-Mediated Immune Effects of MCA Exposure and Tumor Transplantation

The relative reactivities of T and B spleen lymphocytes were determined *in vitro* with spleens removed from mice both during the intratracheal instillation of MCA and 7, 14, and 21 days after transplantation of tumor cells. Spleen lymphocyte responses to PHA, a T-cell specific mitogen, are shown in Fig. 2. Although some depression of T-cell activity occurred during intratracheal instillation of MCA, it reached significant levels only after the second ($p < 0.02$) and fourth (p 0.01) exposures. This depressed response returned to normal within 3 days after instillations of MCA were discontinued. One may observe that CO instillation in itself lowers T-cell activity after the second and third exposures. Most striking in Fig. 2 was the noticeable enhancement of T-cell reactivity produced by both syngeneic and allogeneic tumor cell transplantation regardless of whether the host was previously exposed to MCA or not. T-cell immune activity also was measured in response to allogeneic antigen in mixed lymphocyte cultures, and these results are shown in Fig. 3. Levels of T-cell reactivity were not effected by MCA exposure, but again were noticeably enhanced by transplantation of syngeneic and allogeneic tumor cells.

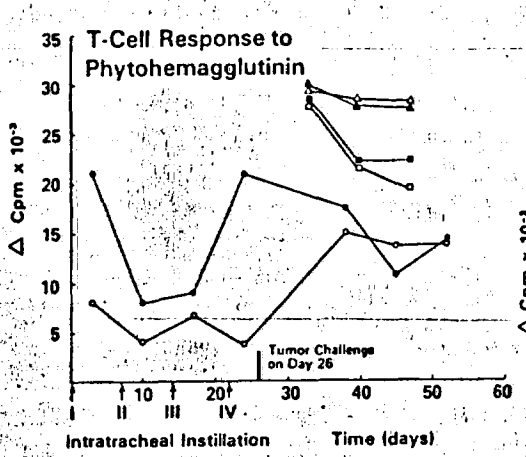

Fig. 2

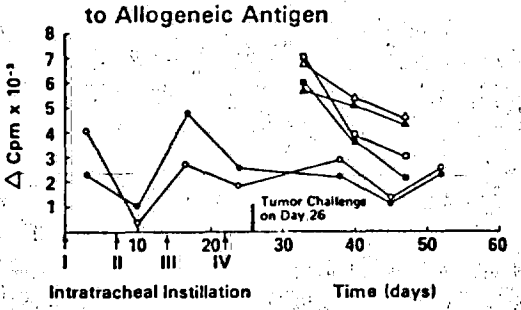

Fig. 3

Fig. 2. Response of spleen lymphocytes to mitogenic stimulation with phytohemagglutinin after intratracheal instillation of corn oil (solid circles) or 3-methylcholanthrene (open circles) and subsequent transplantation of syngeneic (squares) or allogeneic (triangles) tumor cells on day 26. Arrows indicate times of intratracheal instillation at 0, 7, 14, and 21 days

Fig. 3. Response of spleen lymphocytes in mixed lymphocyte culture to allogeneic antigen after intratracheal instillation of corn oil (solid circles) or 3-methylcholanthrene (open circles) and subsequent transplantation of syngeneic (squares) or allogeneic (triangles) tumor cells on day 26. Arrows indicate times of intratracheal instillation at 0, 7, 14, and 21 days

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Whereas T-cell spleen lymphocyte activity was only periodically effected during MCA exposure, bone marrow-derived, B-lymphocyte activity was significantly depressed as shown in Fig. 4. Pronounced levels of depression occurred after instillations at 0 ($p < 0.0005$), 7 ($p < 0.01$), 14 ($p < 0.025$), and 21 ($p < 0.025$) days and remained depressed for 2 weeks after exposures were discontinued. Both T- and B-cell activity were stimulated by syngeneic and allogeneic transplanted tumor cells and again this occurred independent of whether the host had previously been exposed to MCA or not.

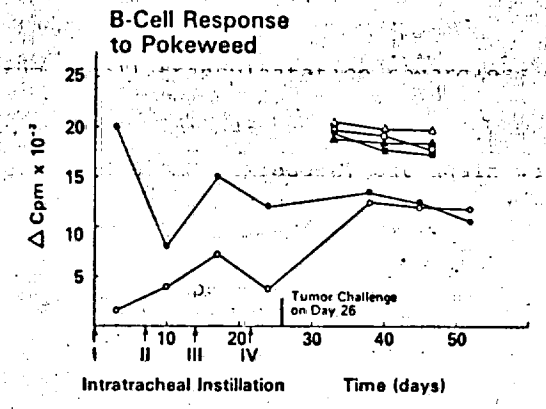

Fig. 4. Response of spleen lymphocytes to stimulation with pokeweed mitogen after intratracheal instillation of corn oil (solid circles) or 3-methylcholanthrene (open circles) and subsequent transplantation of syngeneic (squares) or allogeneic (triangles) tumor cells on day 26. Arrows indicate times of intratracheal instillation at 0, 7, 14, and 21 days

D. Discussion

Contrary to the expectation that MCA exposure might lower host immunocompetence to the point of allowing better growth of transplanted tumor cells, it instead exhibited a tumor-inhibitory effect, at least during the early stages of tumor growth. That transplanted syngeneic tumor cells did not grow as well in mice treated with MCA as in controls treated with CO might be related to the significantly depressed levels of B-cell activity incurred during the intratracheal instillation of MCA. This immunosuppressive effect might have been sufficient to influence at least the early stages of tumor growth. MCA is known to have an immunosuppressive property which impairs the function of B lymphocyte populations and thereby depresses the level of humoral antibody (BALL, 1970; STJERNSWÄRD, 1966; STUTMAN, 1969). Others have shown that antigens elicited on the surface of tumor cells can combine with circulating humoral antibodies to form antigen-antibody complexes effective in the prevention of tumor cell destruction by T-cell effector lymphocytes (BALDWIN et al., 1972, 1973; HELLSTRÖM et al., 1969). This being the case, lower humoral antibody levels in this study as indirectly suggested by the depressed B-cell activity in MCA exposed mice before tumor cell challenge would have permitted a more effective control of tumor growth. However, it is also possible that residual MCA in systemic circulation was simply cytotoxic to transplanted tumor cells and slightly inhibited their growth. Tumor inhibitory effects have been reported by others and have been compared to the deleterious action of x-ray, nitrogen mustard, methotrexate, and other agents on cell proliferation (HUGGINS and MCCARTHY, 1957; THOMPSON et al., 1960). It is difficult to separate the immunosuppressive and cytotoxic capacities

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of various agents (STUTMAN, 1973). Of course, with regard to carcinogenicity, it has already been demonstrated that repeated intratracheal instillation of MCA in mice eventually produces respiratory malignancies (HO and FURST, 1973b; NETTESHEIM and HAMMONS, 1971). To whatever degree mouse immunocompetence was altered in this study, it was not sufficient to overcome a strong histocompatibility barrier and permit growth of transplanted allogeneic tumor cells.

Cell-mediated immune activity as demonstrated by B- and T-cell responses to mitogenic and allogeneic antigen stimulation was clearly enhanced by tumor transplantation regardless of any effect of MCA exposure or tumor growth. The 10% loss of weight by mice exposed to MCA might have been expected to produce a weakened physiological state and a specific loss of immunocompetence. Increased weight of lungs in exposed animals probably was caused by a collection of fluids and infiltration of lymphocytes as an inflamatory response to irritation. The pronounced loss of weight by thymus tissues in response to MCA might well have affected alterations in levels of CMI. Peripheral leukocyte counts were elevated in response to MCA and further reflected chronic irritation of the respiratory tract. However, cell-mediated immune activity was clearly stimulated in response to tumor transplantation and appeared to be independent of any previous MCA induced changes in thymus, lung, spleen, or leukocyte host tissues.

This study suggests that pulmonary exposure to polycyclic hydrocarbons in mice provides a useful model for characterization of the underlying mechanisms of respiratory carcinogenesis and host immunocompetence.

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